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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Aneuploidy is a hallmark of virtually all ovarian cancer cells and is thus indicative of chromosome instability (CIN). CIN may be a preprogrammed strategy of cancer cells to generate enormous genetic and biochemical diversity in a single cell division. CIN therefore allows cancer cells to rapidly evolve and survive suboptimal growth conditions that include drug treatments. The molecular basis of CIN is unclear but we discovered that Aurora B kinase is unable to phosphorylate proteins that are critical for preventing CIN. Restoring Aurora B function can reduce chromosome missegregation in ovarian cancer cells. This strategy should limit their ability to evolve and thus should increase chemosensitivity. We conducted a screen of clinically relevant kinase inhibitors to identify compounds that exhibits off-target effects on the DNA damage checkpoint. Most chemotherapies damage DNA which arrests cells to provide time to repair the damage. Crippling the checkpoint is known to enhance drug sensitivity because cells are forced to enter mitosis with damaged DNA. We identified Bostunib, an FDA approved src/abl inhibitor, as a new checkpoint inhibitor. As compounds specifically designed to inhibit Chk1 were toxic in patients, Bosutinib may provide the first clinically tolerable chemosensitizing agent that targets the DNA damage checkpoint.

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Introduction

The premise of this proposal is rooted in the idea that continual changes in chromosome content of CIN tumor cells provide them with the extraordinary capacity to achieve genetic and biochemical diversities in a single cell division [1-5]. Changes on a global scale may be necessary for tumor cells to adapt to poor growth environments and drug treatments. By understanding the mechanisms responsible for CIN, it should be possible to limit the evolutionary capacity of tumor cells and thus reduce their ability to adapt. By extension, this can be translated to enhanced chemosensitivity. Our preliminary results showed that we had identified a common defect in a panel of 6 ovarian cancer cell lines that explained the molecular basis of their CIN phenotype. Using a combination of high resolution imaging of chromosome dynamics and FISH, we showed defects in the error correction mechanism that normally resolves improper kinetochore:microtubule (kT:MT) attachments. The defect was traced to the inability of the kinetochore-bound pool of the Aurora B kinase to phosphorylate proteins that are associated with defective microtubule attachments. We tested if the problem was due to the inability of Aurora B to gain access to its substrates. By using a CENP-B:INCENP targeting construct to move the endogenous Aurora B closer to its targets, we were able to identify cells that exhibited proper attachments, and did not exhibit missegregation as determined by the absence of lagging chromosomes during anaphase. The salient features of the construct are: 1) CENP-B is used to target the INCENP to a region of the kinetochore that should place it closer to Aurora B substrates. 2) INCENP is moiety that recruits endogenous Aurora B to the recombinant protein.

Body

Task 1. Create and screen recombinant INCENP constructs

- Subclone INCENP and non-INCENP cDNA's into retrovirus vectors

Our initial attempts in expressing the INCENP constructs into retroviral vectors were unsuccessful (no detectable expression). Our troubleshooting efforts led to two alternatives that we are currently implementing. First, we realized that the recombinant INCENP maybe toxic to cells, if the Aurora B is constitutively present at kinetochores throughout the cell cycle (Aurora B is only present at kinetochores during mitosis). Our solution is to add a protease destruction box (D-box) from cyclin B1 to the CENP-B:INCENP fusion. The idea is that the D-box specifies the proteolytic destruction of cyclin B1 at the metaphase to anaphase transition, through ubiquitin mediated proteolysis via the Anaphase Promoting Complex. By including a D-box into our construct, it should be destroyed after each mitosis, and thus reduce any potential toxic side effects it may have if it remained on kinetochores throughout the cell cycle.

The second alteration was to switch from retroviral vectors to lentiviral vectors. Retroviruses do not package large recombinant genomes as effectively as lentiviruses. We recloned the CENP-B-Dbox-INCENP constructs into commercially available pLenti vectors and obtained positive DNAs.

- Transfect constructs into packaging cells to generate recombinant virus

We used the DNAs generated in the past funding period to create recombinant viruses to facilitate their expression in ovarian cancer cells. HEK293 cells were co-transfected with helper plasmids and the INCENP construct. Supernatants were harvested and the cells were harvested for western blotting to verify expression of the recombinant protein (Fig. 1. Lanes 2 and 3).

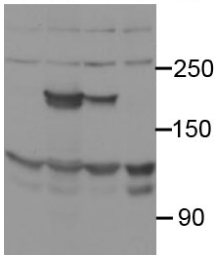

Fig.1. OV10 and PEO1 transfected with CB:INCENP-mCherry(~170kDa). Drug resistant clones of OV10 and PEO1 (lns 1, 4, respectively), or after transient transfection (lns 2, 3) were probed with INCENP. Endogenous INCENP migrates ~120kD.

- Infect OVACAR10 and PE01 cells and screen for drug resistant colonies

The recombinant lentiviruses were used to infect OVCAR 10 and PEO1cells, and we used blasticidin to select for stable integrants. Although resistant clones were isolated (6.5ug/ml), subsequent during the past funding period failed to detect expression (Fig. 1, lanes 1 and 4). We have also tried stepwise selection but we have not obtained positive clones.

- We assume that the CB:INCENP:mCherry may toxic when expressed at the inappropriate levels. We are

cloning the insert into a TetR regulated expression vector. The CMV promoter in this vector contains the TetO elements that block transcription because of the presence of the Tet repressor. Addition of doxycycline or tetracycline to the medium will relieve the inhibition by TetR and allow transcription. A inducible system will allow us to experimentally turn on expression and thus reduce any toxicity issues that are preventing us from obtaining stable clones. This system however requires that we also generate derivatives that express the TetR. Cell lines transfected with TetR will have to be selected and screened for repression expression.

Task 2. Examine chromosome segregation fidelity in INCENP positive cell lines.

- Although failure to isolate clones of stable INCENP expressing OVCAR 3, 5, and 10 cells has hindered our efforts to move the project forward, we have relied on transient transfections to address comments from a manuscript that we had submitted on this subject.

Task 3. Compare tumorogenic properties of CIN parental to non-CIN derivatives.

Pending

Task 4. Inhibit PP1 phosphatase at kinetochores to improve kinetochore functions.

- Our previous efforts to use pharmacological inhibitors of PP1 phosphatase to reduce lagging chromosomes in ovarian cancer cells were unsuccessful. This was likely due to the global effect of the inibitors.
- Our efforts to specifically deplete PP1 from kinetochores by using an siRNA to Sds22 also did not improve error correction. Create and test mutant KNL protein construct that is unable to recruit PP1 to kinetochores.
- We planned to express a KNL mutant that is unable to recruit PP1 to kinetochores, as an alternative to the Sds22 siRNA. Due to issues with using lenti vectors to generate cell lines stably expressing the mutant KNL, current efforts are to try a retroviral system. It is possible that cells cannot tolerate the stable expression of a KNL mutant. This will be resolved in the extension.

Key Research Accomplishments (bulleted list)

- Revising manuscript describing CIN in ovarian cancer cells and rescue by CENP-B:INCENP construct.
- Screened and identified FDA approved Src/Abl inhibitor for off-target effects that can be repurposed for chemosensitization.
- Published mechanism explaining mitotic catastrophe resulting from overriding the DNA damage checkpoint.

Reportable Outcomes

We had to revise a manuscript describing the data presented in the preliminary data of our grant application. This paper presents the novel concept that a localized defect in Aurora B kinase, as opposed to global inactivity, is responsible for the error-correction defects in CIN cells. As we relied exclusively on FRET reporters to compare the localized activity of Aurora B at kinetochores, reviewers demanded larger sample size. We spend the 3 months to increase the datapoints for the cells that were reported, but also had to add a new CIN cell line to the study. We also had to quantitate Aurora B staining at kinetochores to show that the reduced kinase activity was not due to reductions in amounts of kinase at kinetochores. In addition to this, we also quantitated phosphoCENP-A staining, as a readout for Aurora B kinase activity at kinetochores. pCENP-A is phosphorylated by Aurora B but this reaction is not involved in error correction. Quantitation showed that pCENP-A levels were not changed between CIN and non-CIN cells. This reinforced the idea that the defect in error correction is not due to inactivity of the Aurora kinase but is attributed to its failure to access substrates that are critical for error correction. We are expanding the Aurora B substrates that will be subject to quantitative immunofluorescence as we did for pCENP-A. Once this work is completed, it will be resubmitted.

We published a paper, where we showed that inhibitors of the DNA damage checkpoint induce a common mitotic defect that provides the first mechanistic explanation for mitotic catastrophe. As part of our efforts to characterize how inhibitors of the checkpoint were overriding the DNA damage checkpoint, we

discovered a common mitotic defect that results from checkpoint override. Cells arrested in S or G2 with replication and topoII inhibitors, respectively, when forced to enter mitosis, fragment their centromeres from the bulk of the chromatin. This means that genomes lack centromeres that are essential for segregation of chromosomes in subsequent divisions. This centromere fragmentation defect is not seen for radiation induced DSB's. As radiation is presumed to randomly generate breaks in the genome, the chances of fragmenting the centromere is small. However drugs that inhibit replication or topoII inhibitors must induce breaks flanking centromeric chromatin in order to fragment the centromere when cells enter mitosis.

Beeharry N, Rattner J, Caviston J, Yen T. Centromere fragmentation is a common mitotic defect of S and G2 checkpoint override. Cell Cycle 2013; 12:1588 - 1597; PMID: 23624842.
This work will be highlighted in two commentaries that will appear in subsequent issues of *Cell Cycle*.

Our second manuscript describes the ability to repurpose clinically relevant kinase inhibitors, Bosutinib (Bos) and its isomer (Bos-I) to chemosensitize by checkpoint override. Bos and Bos-I are inhibitors of Src/Abl kinases of which Bos has received FDA approval for the treatment of CML. Relying on the promiscuity of kinase inhibitors, we designed a screen to identify compounds with chemosensitization activity. Bos and Bos-I were identified. By screening a database that profiled the effects of 178 drugs against 300 recombinant human protein kinases, we found these compounds to exhibit off target effects to Chk1 and Wee1 kinases. Both of these kinases are targets for chemosensitization. We directly compared the effects of Bos and Bos-I on the in vitro kinase activities of Wee1 and Chk1 and found that Bos was weaker than Bos-I by 11.1 and 3.7-fold, respectively. This was consistent with cell culture experiments that showed higher concentrations of Bos was required to enhance cisplatinin sensitization (Fig. 2). In addition, both drugs sensitized the pancreatic cell lines PANC1, MP-2 and BxPC3 and the ovarian cell lines Ovcar8, A1847 and SKOV-3 to gemcitabine and doxorubicin.

Given that highly potent inhibitors developed for Chk1 have failed in the clinic due to toxicity issues, we are excited by the possibility of using FDA approved drugs to validate chemosensitization in the clinic. Thus, our work may finally lead to clinical validation of the chemosensitization strategy by checkpoint override. This idea would impact treatment of virtually all cancers that are currently being treated with DNA damaging agents as we have reported.

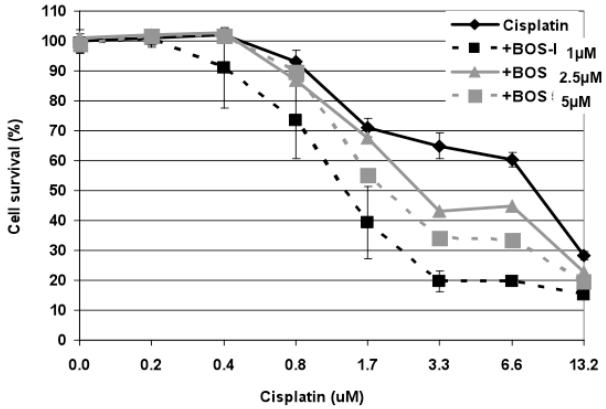

Fig.2. Cisplatinin sensitization by Bos-I and Bos. Cells were treated with different doses of cisplatin alone or with the indicated concentrations of BosI and Bos. Cell viability was assayed 3 days later by standard MTS assay. Data from each treatment condition was normalized to no drug.

Conclusions

Our studies are at a stage where we will test a Tet regulated expression vector that should minimize the toxicity that appears to be associated with constitutive expression of the CB:INCENP:mCherry construct. The alternative is to use the KNL mutant that fails to recruit PP1 to kinetochores to attempt the rescue of the CIN phenotype. Once we can accomplish these goals, we can then continue with the characterization of the cells whose chromosome missegregation rates are hopefully reduced.

Our efforts to repurpose clinically relevant kinase inhibitors as chemosensitizers have identified the src/abl inhibitors Bostunib and its isomer to sensitize a wide variety of cancer cells to commonly used

chemotherapies. Given that Bosutinib is FDA approved, they may provide the first opportunity to test the utility of overriding DNA damage checkpoints in a clinical setting.

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Appendices

None